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ORIGINAL CONTRIBUTION

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Adaptation to chronic ethanol administration emphasized by fatty acid hydroxylations in rat liver and kidney microsomes

Summary *Background* Long-term ethanol consumption in laboratory animals is associated with histological alterations of liver cells and modifications of fatty acid metabolism.

Aim of the study The present study was aimed at investigating the effect of 1- and 2-month chronic treatment of rats with ethanol on the metabolism of two unsaturated (oleic and linoleic) fatty acids in liver and kidney microsomes, in relation to the CYP2E1 enzyme content in both tissues.

Methods Rats were fed ethanol (14 g/Kg/d) or dextrose through a permanently implanted gastric cannula, as described in the intragastric feeding rat model for alcoholic liver disease (ALD). CYP2E1 level was immuno-quantified in both liver and kidney microsomes by Western blot, whereas fatty acid ω- and (ω–1)-hydroxylations were measured using HPLC and radiometric analytical methods.

Results One- and two-month ethanol treatment led to a 3- to 4-fold rise of the CYP2E1 protein in both liver and kidney microsomes. Oleic and linoleic acid (ω–1)-hydroxylations were increased (~3-fold) in liver microsomes after one-month of ethanol administration, but surprisingly such a rise was not observed after a two-month treatment; on the other hand, no effect was observed on the ω-hydroxylations of these fatty acids. Furthermore, as previously described for lauric acid, ethanol intake did not significantly act on the kidney microsome capability to hydroxylate unsaturated fatty acids.

Conclusions CYP2E1 is strongly inducible by ethanol and therefore accounts for the tolerance for this hepatotoxicant. Our results support the development of an adaptation process in the liver hydroxylating enzyme system, which occurs between one and two months of ethanol feeding. Although it is usually not appropriate to extrapolate animal findings to humans, rat and human CYP2E1s were observed to have comparable specificities and similar mechanisms of regulation. Thus, the present study allowed the acquirement of detailed information of CYP2E1 activity in patients with severe manifestations of ALD.

Key Words CYP2E1 – oleic acid – linoleic acid – (ω–1)-hydroxylations – ethanol – adaptation to alcohol

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Introduction

Oleic (C18:1 n–9) and linoleic (C18:2 n–6) acids, two important unsaturated fatty acids in mammals, are partly desaturated and elongated to form derived compounds of essential fatty acids (EFA), which are precursors of molecules of great biological interest. Liver and kidney microsomes in the presence of NADPH oxygenate fatty acids to a large number of metabolites [1–3]. Many of the oxidative systems have been identified as members of the cytochrome P450 superfamily of heme-thiolate enzymes [4]. The microsomal metabolism of oleic acid has been described in rat and human liver, and ethanol is known to effectively induce its (ω–1)-hydroxylation [5]. Linoleic acid and other polyunsaturated fatty acids can be oxygenated by P450s via several pathways: hydroxylation of the ω-sidechain, hydroxylation of bisallylic carbons, epoxidation,

hydroxylation of allylic carbons, or hydroxylation with double-bond migration [3]. Many studies have established the involvement of various P450 isoforms in these reactions [6–8].

Among the P450 family, CYP2E1 is mainly involved in the metabolism of ethanol [9], and its most significant role is the adaptive response to high blood levels with a corresponding acceleration of ethanol metabolism. The metabolic tolerance to ethanol of the heavy drinker is believed to be due to the inducibility of CYP2E1. Ethanol is indeed known to induce CYP2E1 in mammals [10], and its responsibility for alcoholic liver disease (ALD) when associated with high amounts of dietary fat has been established [11]. The CYP2E1 induction is associated with an increased release of reactive oxygen species and of the highly reactive metabolite acetaldehyde, which both play a significant role in the pathogenesis of alcoholic liver injury [9]. Furthermore, CYP2E1 activates many xenobiotics to highly toxic products, explaining the increased vulnerability of the heavy drinker to industrial solvents, drugs, and carcinogens. CYP2E1 is also physiologically involved in the metabolism of lipids and ketones (in starvation, obesity and diabetes) [9]. Chronic administration of ethanol to animals has been described to affect the composition and metabolism of fatty acids in various tissues including the liver. A finding common to these studies is the observation that the concentration of PUFA (polyunsaturated fatty acids), especially arachidonic acid, decreases in the liver [12, 13]. It may be explained by a reduced conversion of linoleate to arachidonate [14]. Moreover, it has also been demonstrated that unsaturated fatty acids such as linoleic acid can potentiate endothelial cell dysfunction through oxidative stress [15]. Furthermore, recent studies in Rhesus monkeys have shown that chronic alcohol exposure may lead to a tissue depletion of PUFA and to an alteration of essential fatty acid metabolism [16].

Oleic and linoleic acids therefore constitute important biological fatty acids; so, it is of high interest to study their microsomal metabolism in the liver after ethanol treatment. Additional data about the role of CYP2E1 induction in the pathogenesis of ALD were reported [17]. The level of CYP2E1 induction is known to be directly related to the CYP2E1-mediated enzymatic activities in ALD. To evaluate the importance of these enzymatic activities in the liver of alcohol-fed animals, the intragastric feeding rat model for ALD [11, 17, 18] was used. The effect of a chronic administration of ethanol over one and two months on the metabolism of these two unsaturated fatty acids in liver and kidney microsomes was investigated in relation to the CYP2E1 protein content. The final purpose of the present study was therefore to adapt the rat CYP2E1 knowledge to humans, and thus to explain some metabolic disturbance observed in ALD.

Materials and methods

Chemicals

Oleic and linoleic acids were purchased from Fluka (Buchs, Switzerland), whereas the radiolabeled molecules [1-^{14}C]oleic acid (50 mCi/mmol) and [1-^{14}C]linoleic acid (48 mCi/mmol) were from Amersham (Amersham, UK). NADPH and 4-nitrophenol (4-NP) were obtained from Sigma (St Quentin Fallavier, France). Electrophoresis reagents were from Bio-Rad (Munich, Germany), whereas nitrocellulose sheets and immunoblot antibodies were supplied by Amersham. All other chemicals and solvents were of analytical grade from Merck (Darmstadt, Germany) or Sigma.

Animals and diets

Each experimental group of animals used for our studies consisted of 5 male Wistar rats weighing about 200 g (Charles River Laboratories, Hollister, CA). All of them were individually fed by continuous infusion through a permanently implanted intragastric cannula as previously described [11, 17, 18]. They were divided into 4 groups: in two of them the rats were chronically pair-fed ethanol for 1 and 2 months, whereas in the last two groups ethanol was isocalorically replaced by dextrose under the same conditions of time. The diets were prepared fresh and contained corn oil contributing to 30–40 % of total calories, as described previously [11, 17]. The amount of liquid diet was adjusted according to the body weight of the alcohol-fed rats as determined weekly. The amount of ethanol administered was initially 6 g/kg/d, and was gradually increased to 14 g/kg/d to maintain high levels of blood alcohol. All the rats were treated in compliance with the Animal Committee of Harbor-UCLA Medical Center for the Care and Use of Laboratory Animals.

At the end of the experiment, liver and kidney samples were removed and immediately frozen in isopentane in liquid nitrogen. The microsomal fraction was prepared according to a previously described method [19]. The subcellular fraction was stored at –80 °C until use in a 100 mM phosphate buffer pH 7.4 containing 20 % glycerol (v/v). Microsomal protein content was determined using the Bradford method (Bio-Rad).

Biochemical and histopathological analysis

Urine alcohol levels were measured on urine collected under toluene over a 24 h period using a QED test (STC Technologies Inc., Bethlehem, PA), while blood alcohol levels were measured using a Radioactive Energy Attenuation Assay (Abbott AXSYN System, Abbott Laboratories, North Chicago, IL).

Alanine aminotransferase (ALT) activity was measured in sera using an automatic analyzer (SYNCH RONCX Systems, Beckman Inst. Inc., Fullerton, CA).

For the determination of the total pathological score, multiple liver sections were examined microscopically, and pathological score was evaluated as previously described [11]. Fatty liver parameter (i.e. the percentage of liver cells containing fat) was determined as follows: < 25 % of cells = 1+, < 50 % = 2+, < 75 % = 3+, and > 75 % = 4+, as previously reported [11].

SDS-Page and immunoblotting of CYP2E1 protein

Aliquots of liver (10 µg) and kidney (30 µg) microsomal samples were separated by electrophoresis on 9 % sodium dodecyl sulfate polyacrylamide gels [20], then electrophoretically transferred to nitrocellulose sheets [21]. The sheets were blocked with TBS containing 3 % (w/v) bovine serum albumin, 10 % fetal calf serum and 0.05 % Tween 20 (v/v) for 30 min at room temperature, and then incubated overnight at 4 °C with polyclonal anti-rat CYP2E1 antibodies (Amersham, UK). After washings with TBS, the nitrocellulose sheets were treated with anti-rabbit immunoglobulin-biotinylated species-specific secondary antibodies conjugated to peroxidase. The blots were developed on photographic films with luminol substrate and hydrogen peroxide of enhanced chemiluminescence (ECL) reagent (Amersham).

In vitro determination of monooxygenase enzymatic activities

4-NP hydroxylation was determined by HPLC according to previously described methods [22, 23].

Oleic and linoleic acid ω- and (ω–1)-hydroxylations were measured by incubating microsomes (0.3 mg protein) in a reaction mixture containing substrate (fatty acid 0.1 mM, 0.5 µCi) in 0.12 M potassium phosphate buffer (pH 7.4) and NADPH 1 mM. The ω- and (ω–1)-hydroxylated metabolites and residual substrate were separated by RP-HPLC using a 5 µm Ultrasphere C18 column, as previously reported [5]. For analysis of radioactivity distribution, samples were developed (2 ml/min) using an initial mobile phase of water/acetonitrile/acetic acid (45:55:0.2; v/v) for 30 min, followed by a linear gradient to 5:95 (v/v) water and acetonitrile for 10 min, in order to elute residual substrates. Radioactivity was routinely monitored with a computerized on-line liquid scintillation counter (Flo-One Packard, Meriden, CT) in presence of scintillation fluid Ultima-Flo AP (Packard) [5, 24].

Furthermore, the ω- and (ω–1)-hydroxylated metabolites of linoleic acid were analyzed and identified by LC/APCI-mass spectrometry on a Navigator LC/MS apparatus (Finnigan, Manchester, UK) equipped with an atmospheric pressure ionization source and running on negative ion mode. The HPLC conditions were the same as described above.

Statistical analysis

Results are expressed as means ± SEM (five animals/group). Differences between groups were evaluated by one-way ANOVA and were considered to be statistically significant when $p < 0.05$.

Results

Treatment of the rats

In all the groups, the rats gained weight at a constant rate (Table 1). No difference in the amount of weight gain among the various groups was observed; these results were in agreement with previously reported data [25, 26]. The blood and urine alcohol levels of the ethanol-fed animals did not significantly differ versus the treatment duration.

The pathological changes in the different experimental groups were similar to those previously described [11, 26, 27]. No significant pathological changes were seen in the dextrose-fed groups. The severity of pathological injury was greater in the ethanol-fed groups than in the dextrose groups; there were no significant differences in the pathological changes observed between the one-month and two-month ethanol-fed groups (Table 1).

Table 1 Blood and urine alcohol levels, weight gain, liver weight, and total pathological score (m ± SEM) in the experimental groups

Experimental group (n = 5)	Blood alcohol level (mg/dL)	Urine alcohol level (mg/dL)	Weight gain (g)	Liver weight (g)	ALT (U/L)		Fatty liver (0–4)	Total pathological score
					baseline*	terminal*		
Dextrose 1 month	0	0	38±6.5	13.1±0.8	49.5±1.8	49.3±9.4	0.6±0.6	1.8±1
Ethanol 1 month	428±36	396±9	39.6±7.1	17.3±1.0	31.8±3.4	22.8±8.4	2.2±0.5	4±1.1
Dextrose 2 months	0	0	89.8±18.3	13.6±1.5	26.6±7.5	32.8±15.4	0	0
Ethanol 2 months	372±55	333±35.5	80.2±21.6	21±1.5	24.2±7.2	30±11.2	3±0.3	4.6±1.1

* baseline: at day 0, terminal: after one-month or two-month treatment

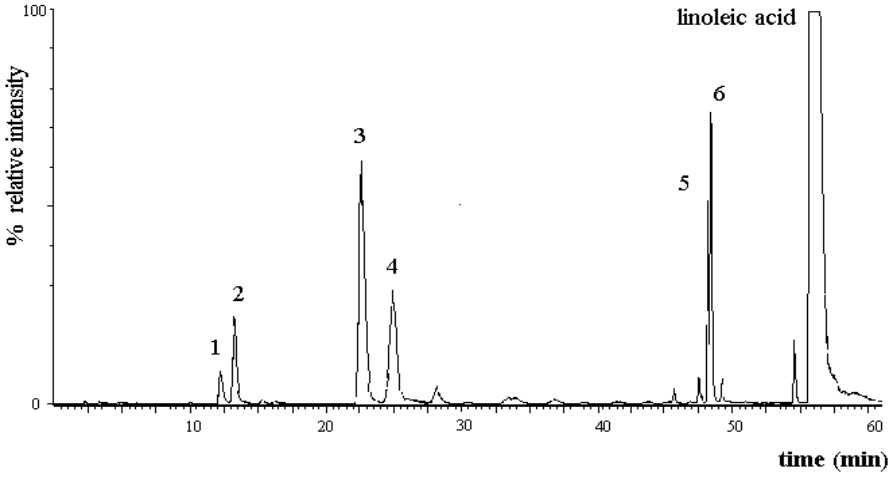

Fig. 1 HPLC separation of linoleic acid metabolites after incubation of microsomes (0.3 mg) from ethanol-treated rats with linoleic acid (0.1 mM). Detection was performed using APCI-mass spectrometry.

Identification of linoleic acid metabolites

Liver and kidney microsomes from dextrose- and ethanol-treated rats were incubated with [^{14}C]-linoleic acid and NADPH. Hydroxylated metabolites were separated and identified according to the method previously described for oleic acid [5]. The separation and identification of the peaks were performed by HPLC coupled to a negative ion APCI-mass spectrometer. Fig. 1 shows that the retention times of the two major hydroxymetabolites (peaks 3 and 4) and residual substrate were 23, 26.5 and 56.5 min, respectively. The generation of these two main metabolites had an absolute requirement for molecular oxygen and NADPH as a source of electrons (data not shown), confirming the involvement of P450 enzymes. These two peaks (3 and 4) were analyzed by LC/MS and characterized by a deprotonated molecule [M-H]- ion at m/z 295 corresponding to the hydroxy-derivative of linoleic acid. The residual substrate exhibited a deprotonated molecule [M-H]- ion at m/z 279. The order of HPLC elution of these two hydroxylated metabolites was (ω-1) and ω-, respectively, as previously reported in numerous studies concerning fatty acids [5, 28]. The other metabolites (peaks 1, 2, 5 and 6) were also analyzed by APCI-mass spectrometry and characterized by a deprotonated molecule [M-H]- ion at m/z 313 for peaks 1 and 2 (corresponding to the mass of a dihydroxy-derivative of linoleic acid), and 295 for peaks 5 and 6 (corresponding to the mass of an epoxy- or hydroxy-derivative of linoleic acid). However, only peaks 3 and 4 were quantified in the study reported here.

Measurement of CYP2E1 protein in liver and kidney microsomes of the experimental groups

Because CYP2E1 is known to be mainly responsible for the (ω–1)-hydroxylation of fatty acids in human and rat liver microsomes [5, 29, 30], the content of specific

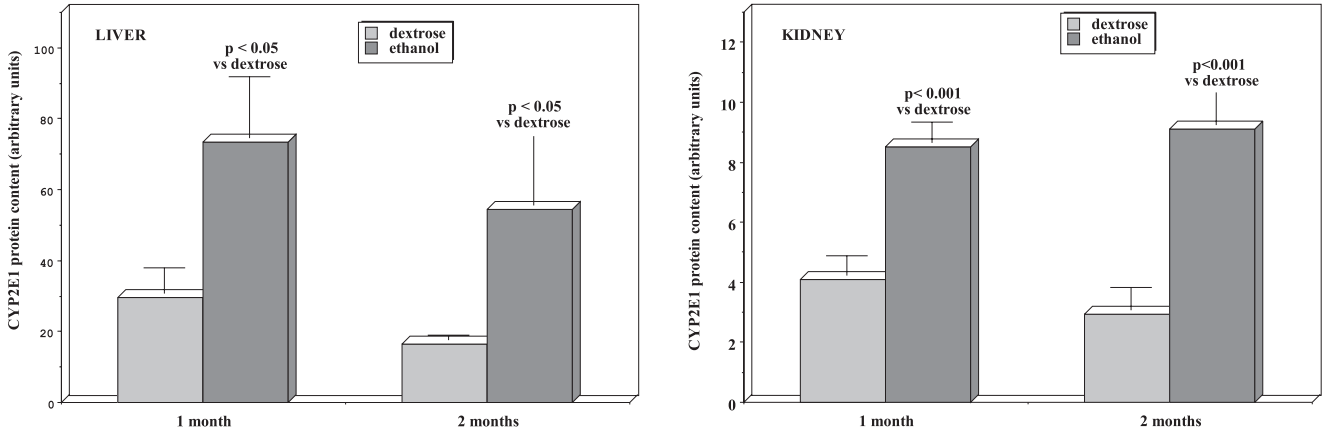

Fig. 2 CYP2E1 protein content in liver and kidney microsomes from control and ethanol-fed rats, as assessed by immunoblot analysis. Values are expressed as arbitrary densitometric units (relative to protein amount) and reported as the means ± SEM.

CYP2E1 isoenzyme was determined by Western Blot analysis in the liver and kidney microsomes of control and ethanol-fed animals.

After administration of ethanol for one month, CYP2E1 analysis (Fig. 2) revealed an increase of the CYP2E1 protein in both liver and kidney microsomes, *i. e.* fold increase of 2.5 ± 0.5 $p < 0.05$ (liver) and 2.3 ± 0.3 $p < 0.001$ (kidney) compared to dextrose. A similar increase was also observed after administration of ethanol for 2 months, *i. e.* fold increase of 3.9 ± 0.5 $p < 0.05$ (liver) and 3.1 ± 0.4 $p < 0.001$ (kidney) relative to dextrose. However in control animals, the microsomes from the liver had a higher content in CYP2E1 protein (approximately 10–15 fold) than those from the kidney.

Monooxygenase enzymatic activities in the different experimental groups

Both the ω- and (ω–1)-hydroxylations of oleic and linoleic acids were measured in liver and kidney microsomes of control and one- and two-month ethanol-fed rats. Their levels were then compared with a monooxygenase enzymatic activity, *i. e.* 4-nitrophenol hydroxylation, known to be CYP2E1 mediated in control and ethanol-fed rats. The data given in Table 2 show that ethanol increased 4-NP hydroxylase activity in the microsomes from the liver (4.6- and 2.4-fold increase further to one- and two-month ethanol feeding respectively) and from the kidney (5.2- and 3.2-fold increase further to one- and two-month ethanol intake).

After one-month ethanol administration oleic and linoleic acid (ω–1)-hydroxylations were increased 3.4- and 3.2-fold for oleic and linoleic acids, respectively. Surprisingly, two-month ethanol treatment did not cause such a rise. The oleate (ω–1)/ω ratio rose from 0.33 ± 0.05 to 0.80 ± 0.09, and from 0.37 ± 0.04 to 0.55 ± 0.05 in liver microsomes of dextrose and ethanol-treated rats for 1 and 2 months, respectively. In the same way, the linoleate (ω–1)/ω ratio passed from 0.44 ± 0.05 to 0.62 ± 0.04, and from 0.52 ± 0.11 to 0.51 ± 0.04 in liver microsomes of dextrose and ethanol-treated rats for 1 and 2 months, respectively. Furthermore, as previously described for lauric acid [30, 31], ethanol had no effect on the ω-hydroxylations of oleic and linoleic acids in liver microsomes.

The ability of the kidney monooxygenase system to respond to ethanol-feeding and that of the liver for 4-NP-hydroxylation were very similar. However, ethanol treatment had no significant effect on the kidney microsomes capability to result in ω- and (ω-1)-hydroxylation of oleic and linoleic acids. No significant difference of the oleate or linoleate (ω-1)/ω ratio was detected in kidney microsomes of dextrose- and ethanol-fed rats.

Discussion

Human and animal studies have demonstrated that chronic administration of ethanol affects the composition and metabolism of fatty acids in most tissues, including the liver [17, 31]. Chronic alcohol exposure is known to lead to significant changes in lipid metabolism, *e. g.* stimulation of the biosynthesis of long-chain polyunsaturated fatty acids in rhesus monkey plasma [16], modification of the fatty acid profile in rat heart tissue [32] or in chick embryo [33] mainly by decreasing the percentage of oleic acid in the

Table 2 Monooxygenase enzymatic activities (m ± SEM) in liver and kidney microsomes from dextrose- and ethanol-fed rats for 1 and 2 months.

Experimental groups		4-NP (nmol/min/mg)	Oleic acid (pmol/min/mg)		Linoleic acid (pmol/min/mg)	
			(ω–1)-OH	ω-OH	(ω–1)-OH	ω-OH
Liver	Dextrose 1 month	760±215	106.7±21.3	329.3±21.3	54.2±7.0	135.6±7.2
	Ethanol 1 month	3460±900	361.1±93.3	396.4±74.9	175.1±53.1	274.7±76.4
		(4.6±1.2)**	(3.4±0.9)*	(1.2±0.2)^{NS}	(3.2±0.7)*	(2.0±0.6)^{NS}
	Dextrose 2 months	880±180	128.4±19.5	351.1±40.1	77.9±14.5	157.6±22.7
	Ethanol 2 months	2100±420	164±26.9	290.7±21.5	83.6±21.2	163.1±34.7
		(2.4±0.5)*	(1.3±0.2)^{NS}	(0.82±0.05)^{NS}	(1.1±0.3)^{NS}	(0.96±0.2)^{NS}
Kidney	Dextrose 1 month	221±54	174.6±8.7	352.4±24.6	85.3±7.7	352±58.0
	Ethanol 1 month	1143±126	196.4±9.4	353.3±72.1	104.4±7.1	358.7±57.8
		(5.2±0.5)***	(1.1±0.05)^{NS}	(1.1±0.15)^{NS}	(1.2±0.1)^{NS}	(1.0±0.15)^{NS}
	Dextrose 2 months	182±26	173.8±9.4	357.8±42.0	92±4.7	296.4±29.5
	Ethanol 2 months	585±161	162.2±31.0	293.9±36.2	85.8±11.1	204.9±55.3
		(3.2±0.9)*	(0.93±0.15)^{NS}	(0.82±0.1)^{NS}	(0.9±0.15)^{NS}	(0.7±0.2)^{NS}

Values in parenthens represent the fold increase (m ± SEM) of enzymatic activities from ethanol-fed rats versus their respective controls.
NS Not statistically significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

phospholipid content of microsomal and mitochondrial membranes. Linoleic acid is known to be metabolized in microsomal preparations through hydroxylation and epoxidation reactions [34].

In the study reported here, liver and kidney microsomes from dextrose- and ethanol-treated rats were found to metabolize oleic and linoleic acids to (ω–1)- and ω-hydroxylated fatty acids as major hydroxymetabolites. These metabolites were identified by LC/MS analysis according to previous experiments [5, 24]. In comparison with dextrose-treated rats, ethanol, a well-known CYP2E1 inducer [29, 35], administered to rats for one month increased by about 3-fold the formation of (ω–1)-OH oleic and linoleic acids, but did not significantly modify the production of ω-OH fatty acids.

The present study also compared the selective CYP2E1 enzymatic activity (4-nitrophenol hydroxylation) and fatty acid hydroxylations in the liver and kidney microsomes of one-month dextrose- or ethanol-fed rats. The levels of induction after ethanol administration differed greatly: indeed, chronic administration of ethanol for one or two months largely increased 4-NP hydroxylase activity in microsomes of both organs, while the formation of (ω–1)-OH oleic and linoleic acids was only increased in liver after one-month ethanol administration. However the fatty acid enzymatic activities were lower than those obtained when lauric acid had been used as substrate [29, 36].

Kidney CYP2E1 protein and enzymatic activities account for approximately 10–15 % of that in the liver [37]. Moreover, CYP2E1 inducers like ethanol regulate CYP2E1 expression by various mechanisms, which depend on the tissue concerned [37]. In the present study, one-month ethanol feeding increased 4-NP hydroxylation by 5.2 and CYP2E1 content by 2.3 in kidney microsomes. However, as previously described for lauric and arachidonic acids in kidney microsomes [38], in the same tissues ethanol-feeding for one or two months did not modify the (ω–1)-hydroxylation of oleic and linoleic acids. These results confirmed that the liver and kidney respond in different ways to ethanol feeding. In the kidney, fatty acid hydroxylase activity was not modulated by the same cytochrome P450 as in the liver [38]. This difference may reflect the different P450 isoform composition of both organs. Indeed, the CYP4A isoforms should be predominant in kidney tissue [39, 40], even after ethanol feeding; on the other hand, the CYP2E1 seems to be the major isoform present in the liver after ethanol-feeding while the CYP4A isoforms are much lower [41, 42]

Our study focused on the comparison of the effect induced by the duration of chronic administration of ethanol on the monooxygenase enzymatic activities in the liver and kidney microsomes of rats. Surprisingly, our results highlighted the occurrence of an adaptation process of some kind in the hydroxylating enzyme system of the liver between one- and two-month regular administrations. Although the CYP2E1 protein level was quite the same after one and two months of ethanol treatment (73.3 ± 12.5 and 54.5 ± 14.4, respectively), it seems that the enzymatic system cannot metabolize the two fatty acids studied. Indeed, oleic and linoleic acid (ω–1)-hydroxylations were significantly increased in liver microsomes after one-month but not two-month ethanol feeding. The inducibility of CYP2E1 protein by chronic alcohol feeding can explain the metabolic tolerance to ethanol in alcoholism and the adaptive increase of ethanol metabolism [9].

The observation of an adaptation-like process emphasized by fatty acid metabolism could be explained by the fact that chronic ethanol consumption by intragastric administration modified the enzymatic activities of the proteasome in rat liver; this change could be related to the degree of oxidative stress induced by ethanol [43, 44]. The CYP2E1 protein was well induced by ethanol, and intragastric ethanol administration did not modify the immunoreactivity of the protein. However, some catalytic sites of the enzyme could be partly inactivated. Moreover, Fataccioli *et al*. [44] observed the reduction of the peptidase activities after 1 month of intragastric administration, but not after voluntary ethanol administration. In our study, the fatty acid metabolism was modified only after one-month feeding.

In conclusion, an adaptive process appeared in the liver after 2 months of chronic ethanol administration, as emphasized by fatty acid (ω–1)-hydroxylase activity; this was not observed in the kidney. This mechanism probably involves a change of the proteasome activity related to the degree of ethanol-induced oxidative stress. This pathway appears to play a pathogenic role in experimental alcoholic liver disease. Although it was usually not appropriate to extrapolate animal findings to humans, the CYP2E1 enzyme was described to be well conserved among mammalian species [45, 46]. Furthermore, the amounts of ethanol administered (14 g/kg/d) to the rats whose metabolism is very active are of the same order than those ingested by chronic alcoholics (7–10 g/kg/d) [9]. Therefore, CYP2E1 constitutes a subfamily in which species extrapolation appears to hold well, and the fatty acid metabolism modification, as observed in chronic ethanol intake, could explain some severe manifestations of ALD.

Abbreviations

EFA: Essential Fatty Acids; PUFA: Polyunsaturated Fatty Acids; P450 or CYP: cytochrome P450 (EC1.14.14.1); ALD: Alcoholic Liver Disease; 4-NP: 4-nitrophenol; ALT: Alanine aminotransferase; LC: Liquid Chromatography; APCI: Atmospheric Pressure Chemical Ionization; LC/MS: Liquid Chromatography/Mass Spectrometry.

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